Form 3

# Abstract of Thesis

| Name ( Darcy Lacanilao Garza ) | |
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| Title | Investigation of the behavior and interaction of ginsenoside Rh2 in model membranes containing cholesterol and sphingomyelin<br><br>(Ginsenoside Rh2のコレステロールとスフィンゴミエリン含有モデル膜における相互作用と物性の解明) |


The 20(*S*)-ginsenoside Rh2 (**Rh2**) is a rare type of triterpenoid saponin from *Panax ginseng* known for its biological effects. Its amphiphilic structure consists of a dammarane backbone and *β*-D-glucose in the 3 position (*Fig. 1*). Contrary to most triterpenoid saponins, Rh2 evokes potency against several cancer cell lines and possesses a similar gross shape to steroidal hormones, which led to the premise that it acts through glucocorticoid receptor (GR). Studies show that Rh2 is capable of increasing the nuclear translocation of GR at par with the activity of the synthetic glucocorticoid, dexamethasone. For a ligand to be recognized by GR located in the plasma membrane or cytoplasm, it has to diffuse through and across the membrane bilayer. Since Rh2 is postulated to adopt a similar mechanism, the investigation of its membrane interaction is deemed crucial. Previous studies suggest that Rh2 requires cholesterol (Cho) and/or sphingomyelin (SM) in its membrane interactions; however, an inductive mechanism has yet to be elucidated. Using biomimetic membranes composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphorylcholine (POPC), N-palmitoyl-D-erythrosphingosylphosphoryl- choline (PSM), and cholesterol (Cho), this research aims to apprehend the lipid- and phase-driven interactions of Rh2 with respect to the totality of the membrane, and at the atomistic scale.

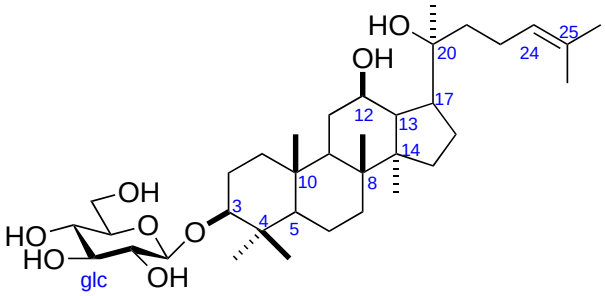


*Figure 1.* Structure of 20(*S*)-ginsenoside Rh2 (**Rh2**)

In the first part of the research, different concentrations of Cho and PSM in models were utilized and compared towards the activity of Rh2. Permeability studies revealed that Rh2 might not exclusively depend on membrane Cho or PSM. This is evidenced by significant leakage activity and membrane deformations of pure POPC vesicles in the presence of Rh2. Experiments at the atomistic level reported the membrane headgroup disordering effects of Rh2 in both Cho and PSM-containing bilayers. In the sample preparation for solid-state NMR, Rh2 was incorporated in the lipid mixture prior to the formation of vesicles; therefore, the saponin is driven towards the membrane interior. The results indicate that Rh2 perturbs interactions at the environment of the phosphocholine headgroup, and decreases junction between lipids. This is presumed to result in splayed acyl chains and reflects in the increased wobbling of deuterium-labelled PSM tail with Rh2 concentration. In contrast, deuterium labelling at the rigid ring of Cho reported limited changes in its tilt angle with increasing Rh2 concentration. This can be rationalized by the constrained interactions of Rh2 with Cho. Rh2' s core has a bifacial rough face enriched with methyl and hydroxyl groups that may restrict its binding with Cho. Furthermore, Cho may prefer mixing with POPC or PSM to uphold the umbrella effect. In the time-course leakage assay, Rh2 was described to have different leakage rates in liquid-ordered and liquid-disordered membranes. Since Rh2 was also

reported to disrupt lipid rafts in cell membranes, its interaction and activity towards different liquid phase states may be noteworthy for investigative research.

In the second study, the behavior of Rh2 towards model membranes with diverse phase states was examined. Based on the phase diagram reported for PSM, POPC, and Cho, lipid bilayers consisting of different lipid mole ratios were prepared to generate homogenous Lo (PSM/POPC/Cho 35:25:40), homogenous Ld (PSM/POPC/Cho 17:75:8), and Lo-dominant phase-separated membranes (PSM/POPC/Cho 1:1:1). Rh2 is a unique saponin that renders diverse effects on different membrane phases. The presence of the hydroxy groups at the 12 and 20 positions allows Rh2 to bind to the polar membrane surface. Based on the fluorescence measurements (DPH, laurdan, and prodan generalized polarization (GP)), a saturation of the membrane surface results in the partial dehydration of the membrane interior and the reinforced hydrophobic interactions of the lipid chains. At higher saponin concentrations, Rh2 inserts effectively into the fluid Ld phase compared to the Lo phase. This insertion is supported by the surface pressure-area ($\pi$-A) isotherms revealing that Rh2 binds more abundantly to the monolayers of the Ld lipid composition than to those of the Lo lipid composition (experiment c/o Dr. Masanao Kinoshita of Kyushu University). In addition, imaging of phase-separated vesicles using confocal fluorescence microscopy illustrates the deformations brought by distinct binding of the saponin in Ld and Lo domains in the presence of Rh2. Molecular Dynamics (MD) simulations also suggest that the sapogenin portion of Rh2 is located in a relatively shallow region of the hydrophobic interior of the Ld phase (*Fig. 2*, experiment c/o Dr. Peter Greimel of RIKEN CBS). These results disclosed the unique mechanism in the efficient membrane permeabilization by Rh2; the saponin accumulates asymmetrically on the surface and in the shallow interior of the less ordered bilayers such as the Ld phase to cause membrane disruption.

Overall, these results disclosed the unique mechanism in the efficient membrane permeabilization by Rh2; the saponin accumulates asymmetrically on the surface and in the shallow interior of the less ordered bilayers such as the Ld phase to cause membrane disruption. Understanding the molecular mechanism of Rh2 is advantageous in ensuring wider applications of the saponin. The importance of determining the precise mechanism of Rh2 concerns its applications, as Rh2 can be devised as an adjuvant in liposomal drug delivery systems. More generally, knowledge of the affinity of saponins for the membrane allows us to modify their structure for improving specific recognition by target cells. A better comprehension of their activities will tailor to factors of drug development, such as structure modification for longer bioavailability and lower hemolytic capacity.

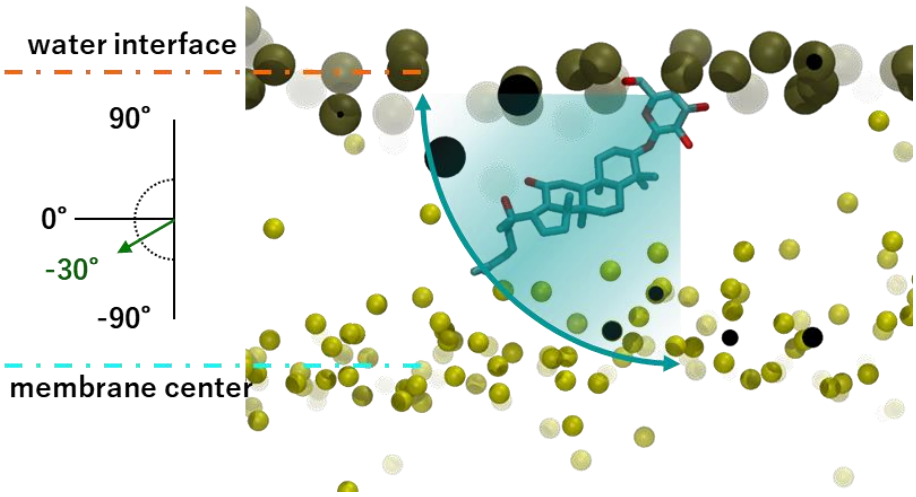


*Figure 2.* Snapshots of MD simulation showing the orientation of Rh2 in bilayers consisting of POPC in the presence of 10 mol% Rh2. The green arrows and degrees in the insertion of the upper panels indicate the orientation of the C3-C17 sterol core of Rh2. The phospholipid headgroups and terminal methyl groups in membranes were shown in large and small balls. Experiment c/o Dr. Peter Greimel of RIKEN CBS.

様式 7

# 論文審査の結果の要旨及び担当者

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トリテルペンやステロールの配糖体であるサポニンは植物に広く分布し、その界面活性作用により細胞膜に結合することによって種々の生物活性を発現する。薬用ニンジンの滋養強壮作用は古くより知られているが、近年では、そのサポニン性有効成分であるginsenoside Rh2 の薬理活性が注目されている。Garza 氏はこれら化合物の細胞膜に対する作用を精密に評価するためにリン脂質二重膜を用い、それらの分子挙動を様々な実験手法によって探究した。Rh2 の膜透過化活性に及ぼす各種脂質の影響を調べたところ、既報の知見とは異なり、コレステロール（Cho）とスフィンゴミエリン（SM）は大きな影響を及ぼさないことがわかった。さらに、脂質の脂肪鎖充填構造と水分子透過性に対する Rh2 の影響を蛍光性分子プローブや固体 NMR によって調べたところ、SM と飽和ホスファチジルコリンを含む二重膜の間で有意な差がないことが分かった。これらの結果は、Rh2 と特定の脂質との特異的な相互作用よりも、液体秩序（Lo）相の形成が Rh2 の膜内での挙動に影響を与えることを示唆した。

そこで、Garza 氏は蛍光性プローブ実験により、主に Cho と SM から成る Lo 相、および主に不飽和脂質から成る液体無秩序（Ld）相に対する Rh2 の影響を調べた。また、共同研究において表面張力測定および分子動力学計算の結果を取得した。表面張力-面積等温線において、Ld と Lo の脂質組成の単層膜を比較したところ、Rh2 はどちらの単層膜にも結合するが、その量は Lo 相よりも Ld 相の方が多いことが判明した。また、主に Lo 相または Ld 相からなる二重膜の水和状態を蛍光性プローブによって測定したところ、Rh2 はどちらの相でも二重膜の表面に結合する傾向が明らかとなり、高濃度では、Rh2 は Lo 相よりも Ld 相の比較的浅い内部に多く結合することがわかった。このような Rh2 の相依存的な膜挙動は、おそらく Rh2 の相選択的な親和性と結合様式に起因すると思われる。このように、Garza 氏は、代表的なサポニンである ginsenoside Rh2 がその薬理活性を示す際に重要な脂質二重膜との相互作用を精査して、既知のサポニンとは異なる分子機構の一端を明らかにした。この成果は、Rh2 の応用研究の基礎となるばかりではなく、膜結合分子の新しい作用様式を示すものであり、基礎科学的にも高い意義を有すると考えられる。

以上の要旨によって、本論文は博士（理学）の学位論文として十分価値あるものと認める。